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(54) Title: COPOLYMER-1 IMPROVEMENTS IN COMPOSITIONS OF COPOLYMERS

(57) Abstract

The present invention relates to an improved composition of copolymer-1 comprising copolymer-1 substantially free of species having a molecular weight of over 40 kilodaltons.

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COPOLYMER-1

IMPROVEMENTS IN COMPOSITIONS OF COPOLYMERS

This application is a continuation-in-part of U.S.S.N. 08/344,248, filed November 23, 1994, which is a continuation of U.S.S.N. 08/248,037, filed May 24, 1994.

Background of the Invention

Copolymer-1 is a synthetic polypeptide analog of myelin basic protein (MBP), which is a natural component of the myelin sheath. It has been suggested as a potential therapeutic agent for multiple sclerosis (Eur. J. Immunol. [1971] 1:242; and J. Neurol. Sci. [1977] 31:433). All references cited herein are hereby incorporated by reference in their entirety. Interest in copolymer-1 as an immunotherapy for multiple sclerosis stems from observations first made in the 1950's that myelin components such as MBP prevent or arrest experimental autoimmune encephalomyelitis (EAE). EAE is a disease resembling multiple sclerosis that can be induced in susceptible animals.

Copolymer-1 was developed by Drs. Sela, Arnon, and their co-workers at the Weizmann Institute (Rehovot, Israel). It was shown to suppress EAE (Eur. J. Immunol. [1971] 1:242; U.S. Patent No. 3,849,550). More recently, copolymer-1 was shown to be beneficial for patients with the exacerbating-remitting form of multiple sclerosis (N. Engl. J. Med. [1987] 317:408). Patients treated with daily injections of copolymer-1 had fewer exacerbations and smaller increases in their disability status than the control patients.

Copolymer-1 is a mixture of polypeptides composed of alanine, glutamic acid, lysine, and tyrosine in a molar

ratio of approximately 6:2:5:1, respectively. It is synthesized by chemically polymerizing the four amino acids forming products with average molecular weights of 23,000 daltons (U.S. Patent No. 3,849,550).

It is an object of the present invention to provide an improved composition of copolymer-1.

Summary of the Invention

The present invention relates to a composition of copolymer-1 substantially free of species of copolymer-1 having a molecular weight of over 40 kilodaltons (KDa).

The invention further relates to a copolymer-1 having over 75% of its molar fraction within the molecular weight range from about 2 KDa to about 20 KDa.

In addition, the invention relates to a copolymer-1 having an average molecular weight of about 4 to about 8.6 KDa.

Moreover, the invention relates to a pharmaceutical composition and a method for the treatment of multiple sclerosis, using the above-discussed copolymer-1.

Brief Description of the Drawings

Figure 1 displays the molecular weight distribution of three batches of copolymer-1, showing the proportion of species with molecular weight above 40KDa. Figure 2 shows similar data relating to the molar fraction.

Detailed Description of the Invention

The present invention relates to a composition of copolymer-1 substantially free of species of copolymer-1 having a molecular weight of over 40 kilodaltons (KDa). Preferably, the composition contains less than 5% of species of copolymer-1 having a molecular weight of 40

KDa or more. More preferably, the composition contains less than 2.5% of species of copolymer-1 having a molecular weight of 40 KDa, or more.

The invention further relates to a copolymer-1 having over 75% of its molar fraction within the molecular weight range from about 2 KDa to about 20 KDa.

In addition, the invention relates to a copolymer-1 having an average molecular weight of about 4 to about 8.6 KDa. In particular, the invention relates to a copolymer-1 having an average molecular weight of about 4 to about 8 KDa and a copolymer-1 having an average molecular weight of about 6.25 to about 8.4 KDa.

Copolymer-1, according to the present invention, may be prepared by methods known in the art, for example, the process disclosed in U.S. Patent 3,849,550, wherein the N-carboxyanhydrides of tyrosine, alanine, y-benzyl glutamate and E-N-trifluoro-acetyllysine are polymerised at ambient temperature in anhydrous dioxane with diethylamine as initiator. The deblocking of the y-carboxyl group of the glutamic acid is effected by hydrogen bromide in glacial acetic acid and is followed by the removal of the trifluoroacetyl groups from the lysine residues by 1M piperidine. For the purposes of the application, the terms "ambient temperature" and "room temperature" should be understood to mean a temperature ranging from about 20 to about 26 °C.

The copolymer-1 with the required molecular weight profile can be obtained either by methods known per se. Such methods include chromatography of copolymer-1 containing high molecular weight species and collecting the fractions without the undesired species or by partial acid or enzymatic hydrolysis to remove the high molecular weight species with subsequent purification by dialysis

or ultrafiltration. A further method to obtain copolymer-1 with the desired molecular weight profile is by preparing the desired species while the amino acids are still protected and then obtain the correct species directly upon removing the protection. The compositions of the present invention may be formulated by conventional methods known in the art. Preferably, the composition is lyophilized and formed into an aqueous solution suitable for sub-cutaneous injection. Alternatively, copolymer-1 may be formulated in any of the forms known in the art for preparing oral, nasal, buccal, or rectal formulations of peptide drugs.

Typically, copolymer-1 is administered daily to patients suffering from multiple sclerosis at a dosage of 20mg.

The invention will be exemplified but not necessarily limited by the following Examples.

EXAMPLE 1

Chromatographic method of preparation of low-toxicity copolymer-1 Two batches of copolymer-1 were prepared according to the methods known in the art, for example, U.S. Patent No. 3,849,550.

One batch was then subjected to chromatographic separation, as described below.

A column for gel filtration, FRACTOGEL TSK HW55 (600 x 26mm) was prepared in a Superformance 26 Merck cartridge according to the manufacturer's instructions. The column was equilibrated with water and acetone solution was injected for total volume determination. The column was equilibrated with 0.2M ammonium acetate buffer pH 5.0. 30 ml copolymer-1 samples (20mg/ml, in 0.2M ammonium acetate pH 5.0) were loaded on the column and fractions were collected every 10 minutes. A fraction having an

average molecular weight of 7-8 KDa was isolated between 120-130 minutes (Batch A).

Molecular Weight Analysis

UV absorbance at 275 nm was determined in a UVIKON 810 spectrophotometer. Samples were diluted to obtain a UV absorbance lower than 1 Absorption Unit. The molecular distribution of the 2 batches was determined on a calibrated gel filtration column (Superose 12).

Copolymer-1 batch A was found to have an average molecular weight of 7-8 KDa. 2.5% of this batch had a molecular weight above 32 KDa, but no copolymer-1 species present in this batch had a molecular weight of over 40 KDa.

The other batch of copolymer-1 which was not subjected to chromatography, had an average molecular weight of 12 KDa. 2.5% of the batch had a molecular weight above 42KDa and 5% of the total copolymer-1 species in this batch had a molecular weight of over 40 KDa.

EXAMPLE 2

Toxicity Analysis

A: In Vivo

Three batches of copolymer-1 having an average molecular weight of 7.3 and 8.4 KDa (less than 2.5% copolymer-1 species over 40KDa) and 22KDa (more than 5% copolymer-1 species over 40KDa) were subjected to the toxicity test described below. In each case 5 mice were used in each experimental group.

Method

Copolymer-1 was dissolved in distilled water to yield a solution of 2mg/ml of the active ingredient. Each mouse was injected with 0.5ml of the test solution into the lateral tail vein. Mice were observed for mortality and

relevant clinical signs over a 48 hour period. Observations were recorded 10 minutes, 24 hours and 48 hours post-injection. If, at the end of 48 hours, all the animals were alive and no adverse signs had been observed, then the batch was designated "non-toxic". If, however, one or more of the mice had died or had shown adverse signs, then the batch was designated "toxic".

The batches with the average molecular weight of 7.3 and 8.4 KDa were both designated "non-toxic", whereas in the batch with the average molecular weight of 22KDa, 3 out of 5 mice had died at the end of 48 hours, and it was consequently designated "toxic".

B: In Vitro

RBL - Degranulation test

I. Introduction

Histamine (or serotonin) release from basophile is an in vitro model for immediate hypersensitivity. The Rat Basophilic Leukemia cell line (RBL-$2H_3$) was developed and characterized as a highly sensitive, uniform, easy to maintain in culture and reproducible system (E.L. Basumian, C. Isersky, M.G. Petrino and R.P. Siraganian. Eur. J. Immunol. 11, 317 (1981)). The physiological stimulus for histamine release involves binding of the antigen to membrane-bound IgE molecules, resulting in the latter's cross-linking and the consequent triggering of an intricate biochemical cascade. Beside these physiological, immunoglobulin-mediated triggers, degranulation can be induced by different non-IgE-mediated stimuli. Among these are various peptides and synthetic polymers, e.g. polylysine (R.P. Siraganian. Trends in Pharmacological Sciences, October 432 (1983)). The RBL degranulation test is, therefore, used in order to screen out those batches of copolymer-1 which evoke substantial degranulation and thus might elicit undesirable local and/or systemic side effects.

II. Principle of the test method

Rat Basophilic Leukemia cells (RBL-2H$_3$), are loaded with [^{3}H]-serotonin, followed by incubation with 100 μg of the copolymer-1 to be tested. Batches of copolymer-1 which induce non-specific degranulation, release [^{3}H]-serotonin into the medium. The radioactivity in the medium is counted by a scintillation counter and the total radiolabeled serotonin incorporated into the cells is determined in the pelleted cells. Percent degranulation is calculated as the percentage of serotonin released out of the total incorporated.

III. Results

Four batches of copolymer-1, with average molecular weight between 6,250-14,500 were analyzed for both % of the species with molecular weight over 40KDa and for degranulation of RBL's. Results are summarized in the following table.

Average M.W. (Daltons)	% of species with M.W. over 40KDa	% Serotonin Release
6,250	< 2.5	12.4
7,300	< 2.5	21.0
13,000	> 5	66.9
14,500	> 5	67.8

As can be seen, when the % of high molecular weight species is low (< 2.5), the % release of serotonin, indicative of toxicity, is low, and vice versa.

EXAMPLE 3

Preparation of Trifluoroacetyl-Copolymer-1

Protected copolymer-1 is prepared as described by Teitelbaum et al. Eur. J. Immun. Vol. 1 p. 242 (1971) from the N-carboxyanhydrides of tyrosine (18g), alanine (50g), y-benzyl glutamate (35g) and trifluoroacetyllysine

(83g) dissolved in 3.5 liters of dioxane.

The polymerization process is initiated by the addition of 0.01 - 0.02% diethylamine. The reaction mixture is stirred at room temperature for 24 hours and then poured into 10 liters water. The product (protected copolymer-1) is filtered, washed with water and dried. The removal of the gamma-benzyl blocking groups from the glutamate residue is carried out by treating the protected copolymer-1 with 33% hydrobromic acid in glacial acetic acid at room temperature for 6-12 hours with stirring. The product is poured into excess water, filtered, washed and dried, yielding the trifluoroacetyl-copolymer-1.

EXAMPLE 4

Preparation of Trifluoroacetyl-Copolymer-1

Protected copolymer-1 is prepared as described by Teitelbaum et al. Eur. J. Immun. Vol. 1 p. 242 (1971) from the N-carboxyanhydrides of tyrosine (18g), alanine (50g), γ-benzyl glutamate (35g) and trifluoroacetyllysine (83g) dissolved in 3.5 liters of dioxane.

The polymerization process is initiated by the addition of 0.01 - 0.02% diethylamine. The reaction mixture is stirred at room temperature for 24 hours and then poured into 10 liters water. The product (protected copolymer-1) is filtered, washed with water and dried.

Protected copolymer-1 is treated with 33% HBr in acetic acid which removes the omega benzyl protecting group from the 5-carboxylate of the glutamate residue and cleaves the polymer to smaller polypeptides. The time needed for obtaining copolymer-1 of molecular weight 7,000±2,000 Da depends on the reaction temperature and the size of protected copolymer-1. At temperatures of between 20-28°C a test reaction is performed on every batch at different time periods for example, from 10-50 hours.

The results concerning the molecular weights of these small scale reactions are calculated and a curve of molecular weight against time is drawn. The time needed for obtaining molecular weight 7,000±2,000 Da is calculated from the curve and performed in larger scale reaction. On average, working at 26°C the time period is 17 hours. The product is poured into excess water, filtered, washed and dried, yielding the trifluoroacetyl-copolymer-1.

Preparation of low-toxicity copolymer-1

20g of trifluoroacetyl-copolymer-1 are dispersed in 1 liter of water to which 100g piperidine are added. The mixture is stirred for 24 hours at room temperature and filtered. The solution of crude copolymer-1 is distributed into dialysis bags and dialyzed at 10°-20°C against water until a pH = 8 is attained. It is then dialyzed against about 0.3% acetic acid and again water until a pH = 5.5-6.0 is obtained. This solution is then concentrated and lyophilized to dryness.

CLAIMS

1. A copolymer-1 fraction substantially free of species of copolymer-1 having a molecular weight of over 40 kilodaltons.

2. A copolymer-1 fraction, wherein said fraction contains less than 5% of species of copolymer-1 having a molecular weight of over 40 kilodaltons.

3. The copolymer-1 fraction according to claim 2, wherein the fraction contains less than 2.5% of species of copolymer-1 having a molecular weight of over 40 kilodaltons.

4. The copolymer-1 fraction according to claim 2, wherein over 75% of said fraction is within a molecular weight range from about 2 kilodaltons to about 20 kilodaltons.

5. A copolymer-1 fraction, wherein said copolymer-1 has an average molecular weight of about 4 to about 8 kilodaltons.

6. A copolymer-1 fraction, wherein said copolymer-1 has an average molecular weight of about 6.25 to about 8.4 kilodaltons.

7. A composition for the treatment of multiple sclerosis, comprising

a pharmaceutically effective amount of a copolymer-1 fraction, wherein said fraction contains less than 5% of species of copolymer-1 having a molecular weight of over 40 kilodaltons, and

a pharmaceutically acceptable carrier.

8. The composition according to claim 7, wherein the

copolymer-1 fraction contains less than 2.5% of species of copolymer-1 having a molecular weight of over 40 kilodaltons.

9. The composition according to claim 7, wherein over 75% of said copolymer-1 in said fraction is within a molecular weight range of about 2 kilodaltons to about 20 kilodaltons.

10. A composition for the treatment of multiple sclerosis, comprising

a pharmaceutically effective amount of a copolymer-1 fraction, wherein said copolymer-1 in said fraction has an average molecular weight of about 4 to about 8 kilodaltons, and

a pharmaceutically acceptable carrier.

11. A composition for the treatment of multiple sclerosis, comprising

a pharmaceutically effective amount of a copolymer-1 fraction, wherein said copolymer-1 in said fraction has an average molecular weight of about 6.25 to about 8.4 kilodaltons, and

a pharmaceutically acceptable carrier.

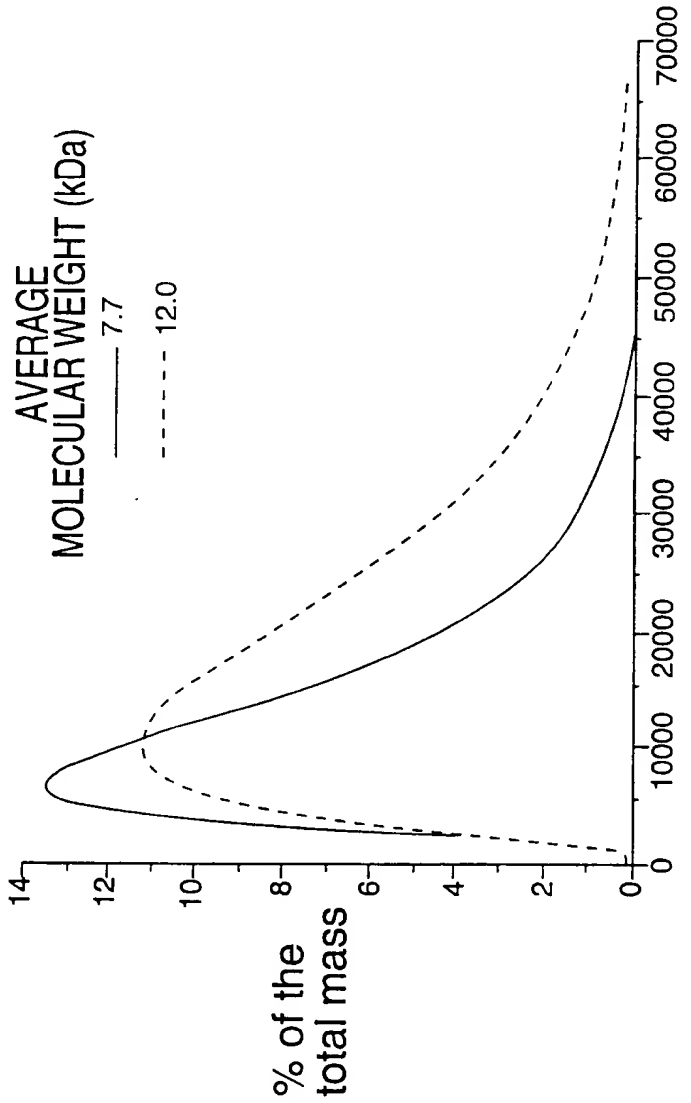
AVERAGE
MOLECULAR WEIGHT (kDa)
7.7
12.0
% of the
total mass
0
2
4
6
8
10
12
14
0
10000
20000
30000
40000
50000
60000
70000
Molecular Weight

FIG. 1

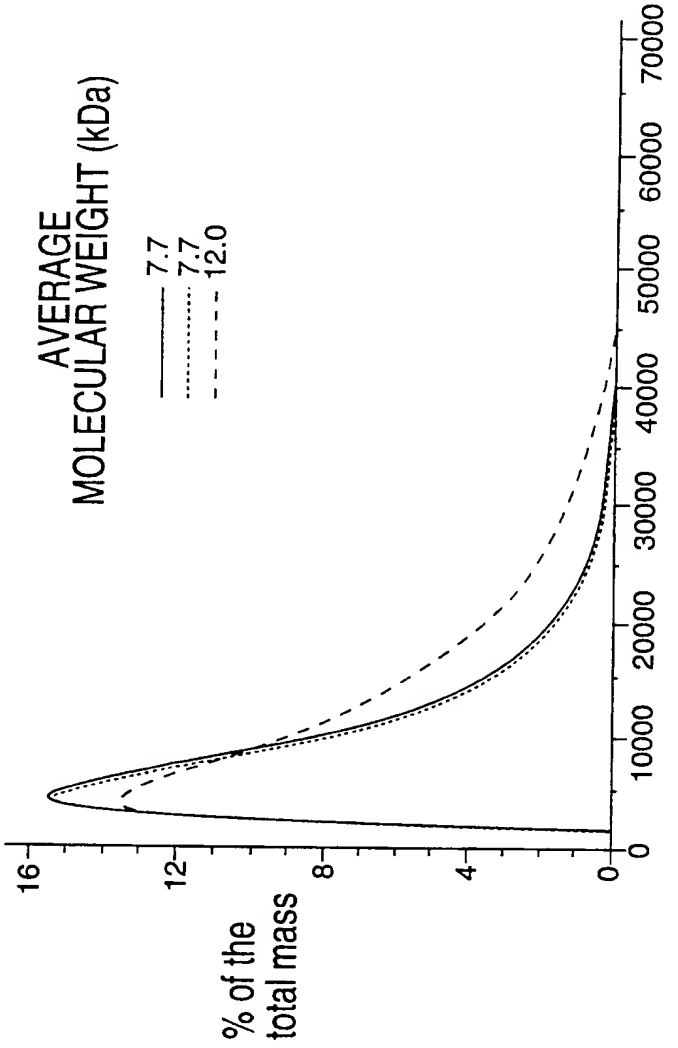
AVERAGE
MOLECULAR WEIGHT (kDa)
7.7
7.7
12.0
% of the
total mass
16
12
8
4
0
0
10000
20000
30000
40000
50000
60000
70000
Molecular Weight

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06551

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :Please See Extra Sheet.
US CL :424/78.29, 78.08, 78.26; 514/561; 525/420, 434, 435; 528/328
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/78.29, 78.08, 78.26; 514/561; 525/420, 434, 435; 528/328

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	EP,A, 0,383,620 (COOK) 22 August 1990, see entire document.	1 ---- 2-11
X ---- Y	US,A, 3,849,550 (TEITELBAUM ET AL.) 19 November 1974, see entire document.	1 ----- 2-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

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